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Fos imaging reveals differential neuronal activation of areas of rat temporal cortex by novel and familiar sounds

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Abstract

To provide information about the possible regions involved in auditory recognition memory, this study employed an imaging technique that has proved valuable in the study of visual recognition memory. The technique was used to image populations of neurons that are differentially activated by novel and familiar auditory stimuli, thereby paralleling previous studies of visual familiarity discrimination. Differences evoked by novel and familiar sounds in the activation of neurons were measured in different parts of the rat auditory pathway by immunohistochemistry for the protein product (Fos) of the immediate early gene *c-fos*. Significantly higher counts of stained neuronal nuclei ($266 \pm 21/\text{mm}^2$) were evoked by novel than by familiar sounds ($192 \pm 17/\text{mm}^2$) in the auditory association cortex (area Te3; AudA). No such significant differences were found for the inferior colliculus, primary auditory cortex, postrhinal cortex, perirhinal cortex (PRH), entorhinal cortex, amygdala or hippocampus. These findings are discussed in relation to the results of lesion studies and what is known of areas involved in familiarity discrimination for visual stimuli. Differential activation is produced by novel and familiar individual stimuli in sensory association cortex for both auditory and visual stimuli, whereas the PRH is differentially activated by visual but not auditory stimuli. It is suggested that this latter difference is related to the nature of the particular auditory and visual stimuli used.

Introduction

Electrophysiological and ablation studies in primates and rats have established that the perirhinal cortex (PRH) of the temporal lobe plays a crucial role in visual recognition memory tasks where the task may be solved by discriminating the familiarity or recency of individual items (Gaffan & Murray, 1992; Suzuki *et al.*, 1993; Mumby & Pinel, 1994; Zhu *et al.*, 1995a; Eichenbaum *et al.*, 1996; Ennaceur *et al.*, 1996b; Murray, 1996; Suzuki, 1996b; Brown & Xiang, 1998; Murray & Bussey, 1999; Brown & Aggleton, 2001). It is important to establish whether the PRH has an equivalent role in recognition memory for all stimulus modalities and not merely for visual stimuli. It is plausible that this should be so as the PRH receives afferent information from all the sensory systems (Felleman & Van Essen, 1991; Burwell *et al.*, 1995; Shi & Cassell, 1997; Burwell & Amaral, 1998). Moreover, recognition memory deficits for somatic sensory and for olfactory stimuli follow lesions of the PRH (Otto & Eichenbaum, 1992; Suzuki *et al.*, 1993). Nevertheless, recent work has suggested that auditory stimuli could provide an exception: in two experiments, one in the dog and the other in the monkey, no significant impairment of recognition memory for auditory stimuli was found after combined perirhinal and entorhinal lesions (Kowalska & Kuśmierek, 1997; Saunders *et al.*, 1998; Kowalska *et al.*, 2001). Determining that auditory stimuli are an exception would provide a potential challenge to the current understanding of the neural substrates of recognition memory.

A series of studies conducted under closely controlled conditions established that it is possible to image rat brain regions involved in familiarity discrimination for visual stimuli using immunohistochemistry for the protein products (Fos) of the immediate early gene *c-fos* (Zhu *et al.*, 1995b, 1996; Wan *et al.*, 1999a). By using Fos as a marker for the differential activation of neurons (Dragunow, 1996; Herdegen & Leah, 1998), these studies showed significantly greater neuronal activation by novel than by familiar individual visual stimuli in the PRH and neighbouring visual association cortex (area Te2), findings which are in accordance with those of ablation and single neuronal recording experiments (Brown & Xiang, 1998; Murray & Bussey, 1999; Brown & Aggleton, 2001). It was therefore decided to use this immunohistochemical technique to image the neuronal activation produced by novel and familiar sounds within the rat brain. If processing for auditory stimuli parallels that for visual stimuli, there should be greater activation for novel than familiar sounds in the auditory association cortex and PRH. To this end, certain sounds were made familiar to a rat by repeated exposure over several days in a procedure similar to that used in the visual experiments, so that the activation produced by these repeatedly experienced sounds could be compared to that produced by a series of sounds which were being heard for the first time.

Previous anatomical and physiological studies of rat auditory systems have established the locations of the primary auditory cortex (AudP; Te1) and association auditory areas (Te3), that the rat can hear sounds in the range 250 Hz to 70 kHz, and that conditioning can produce physiological changes in auditory cortex (Kelly & Masterton, 1977; Roger & Arnault, 1989; Romanski & LeDoux, 1993a, b; Heffner *et al.*, 1994; Friauf, 1995; Scheich *et al.*, 1997; Shi

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& Cassell, 1997; Poremba *et al.*, 1998). Using this information, the sampled areas in the present study included those that might be expected to be activated by auditory stimuli and/or which might be involved in auditory recognition memory, namely the inferior colliculus (IC), AudP and association auditory cortex (AudA), PRH, postrhinal cortex (PoRH), the amygdala (Amyg) and parts of the hippocampal formation (HPC). Preliminary findings have been published in abstract form (Wan *et al.*, 1999b).

Materials and methods

Animals and apparatus

A total of 22 male, pigmented rats (DA strain; Bantin and Kingman, Hull, UK) weighing from 155 to 185 g were used. All animal procedures were performed in accordance with United Kingdom Animals Scientific Procedures Act (1986) and associated guidelines. For at least 1 week before training started, the animals were kept on a 12 h light : 12 h dark cycle with lights off at 0.700 h. Each rat was trained in a perspex chamber (30 × 30 × 35 cm) with an open top. The chamber was situated in a large, dimly lit (1 Lux) matt black, metal box (80 cm × 90 cm × 100 cm). Additional infrared illumination (15 W bulb, with Kodak Beehive Safelamp, Wratten Series 2, Kodak Ltd, London, UK) allowed the continuous observation of the rat's behaviour by means of a TV camera (VISTA, NCD 360, Japan; this behaviour was also video-recorded). The front wall of the chamber was translucent perspex whereas other sides were painted black. A hole in the middle of the front wall (6 cm above the base, 3 cm in diameter) permitted the rat to reach and lick a metal tube that delivered drops of diluted blackcurrant juice. The tube was located outside the chamber, 11 cm from the hole. Interruption of an infrared beam by a rat's head positioned in the hole was detected by the computer. Two loudspeakers (Typhoon Sound System, Model PS-102, Taiwan) were located 20 cm to each side and 25 cm in front of the hole. The programming of trial events, presentation of stimuli, data collection and juice delivery were controlled by a computer (Viglen Pentium PC, Model No. AX1595, Viglen Ltd, Middlesex, UK) in conjunction with an interface (1401plus, CED, Cambridge, UK) and a sound card (ES1868 ESS Technology Inc., Taiwan; frequency response 40 Hz–20 kHz).

Stimuli

The stimuli were computer digitized (sampling frequency 22.05 kHz, 16-bit resolution) records of complex sounds of, for example, trains, cars, musical instruments, animal vocalizations and various items being dropped or hit (see Table 1 for a complete list). The sounds were amplified to 50–85 dBA at the rat's head position, and each lasted approximately 3 s. Two sets, each of 30 sounds, were selected as the familiar and novel sets to be used on the test day. Two other sets of 30 sounds were also used as 'practice' novel sets during training.

Behavioural procedure

Pretraining followed that described in Wan *et al.* (1999a). The rats were allowed access to water for 2 h a day. Each subject was pretrained to hold its head in the hole and receive juice. Training then lasted 6 days, with a morning session and an afternoon session each day. The start of the afternoon session was separated from the end of the morning session by 3 h. A trial started when a rat's head remained positioned in the hole for 1–2 s; then, a stimulus was presented. Towards the end of the sound a drop of juice was delivered through

TABLE 1. Sounds used as the test novel and familiar sets

Set A	Set D
1. Ambulance siren	1. Storm
2. Baby crying	2. Chopping wood
3. Vibrations of a spring	3. Castanets
4. Two bears calling	4. Helicopter
5. Ten-pin bowling	5. Coughing
6. Wind blowing	6. Cow mooing
7. Church bells	7. Glass breaking
8. Cicada	8. Car crash
9. Man speaking	9. Cricket chirping
10. Drilling	10. Crow
11. Waterfall	11. Cuckoo
12. Fire	12. Doorbell
13. Gong	13. Elephant trumpeting
14. Fireworks	14. Flute
15. Horse neighing	15. Japanese folk music
16. Wolf howling	16. Frog croaking
17. Car horn	17. Hammering
18. Lions roaring	18. High-speed train
19. Mosquito	19. Vibrating object
20. Pig	20. Harp
21. Snooker balls colliding	21. Laughter
22. Fire siren	22. Police siren
23. Earthquake	23. Phone ringing
24. Sitar music	24. Ping-pong balls
25. Sawing	25. Ripples
26. Blowing into a shell	26. Spraying water
27. Pouring water into a sink	27. People speaking
28. Steam engine starting	28. Temple sounds
29. Sneezing	29. Wind chimes
30. Pouring liquid	30. Yawning

The use of sounds as the test novel or familiar set was counterbalanced across rats. The sets are available on request from the corresponding author.

the metal tube. After a post-trial delay of 2.5 s, the next trial could start.

In each morning session, the animals heard twice the set of sounds that was to become the familiar set. In the afternoon session, half the rats heard sets of novel sounds on days 1, 3 and 5 (each set was different) and the familiar set of sounds on days 2, 4 and 6. The other half of the rats heard the three sets of novel sounds on days 2, 4 and 6, and the set of familiar sounds on days 1, 3 and 5. The order of stimuli within the familiar set changed from presentation to presentation. Therefore, by the end of the afternoon on day 6 (the test day) all rats had heard an equal number of novel and familiar sounds. However, on the afternoon session of day 6, 11 rats heard the familiar set of sounds for the fifteenth time and 11 heard the test novel set for the first time. All rats completed this final afternoon session in 5–10 min. The familiar set of sounds for one subject was the novel set for the next subject, so that the sounds used as novel or familiar were counterbalanced across the subjects. After removal from their home cage, when the animals were not being trained or tested, they were kept in the dark in a holding cage in a separate, quiet room, aurally isolated from the testing room.

Tissue-processing procedure and data analysis

On the afternoon of the test day, 1.5 h after presentation of the auditory stimuli, each rat was deeply anaesthetized with pentobarbital and perfused with 0.1 M phosphate buffer and 4% paraformaldehyde, pH 7.4. After perfusion, the brain was removed and placed for 12 h in 4% paraformaldehyde, followed by 24–36 h in 30% sucrose in phosphate buffer until the brain sank. The tissue-processing procedure followed that described by Zhu *et al.* (1995b).

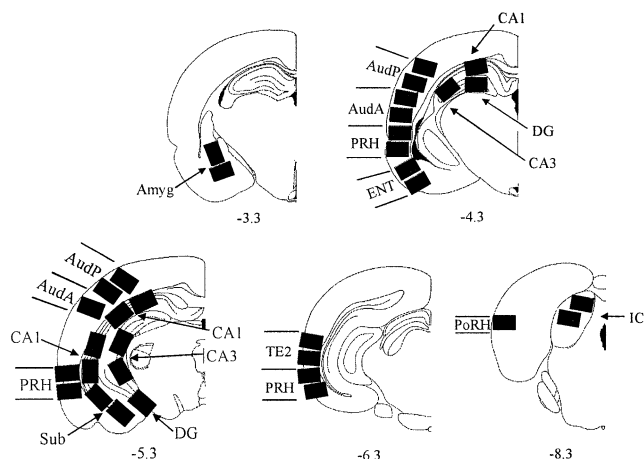


FIG. 1. Sampled areas are shown on sections at the indicated distances (in mm) behind bregma (Paxinos & Watson, 1986). Amyg (amygdala), AudP (primary auditory cortex; Te1), AudA (auditory association cortex; Te3), CA1, CA3 and DG (subfields of hippocampal formation), ENT (entorhinal cortex), HPC (hippocampal formation), IC (inferior colliculus, external nucleus), MG (medial geniculate), PoRH (postrhinal cortex), PRH (postrhinal cortex), Sub (subiculum of hippocampal formation), TE2 (visual association cortex).

Briefly, coronal sections (30 μ m) were cut in a cryostat, and floating sections were processed using a primary antibody and the avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA, USA). The primary antibody was a generous gift of Dr D. Hancock (Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Institute, London, UK) and was a rabbit polyclonal directed against the N-terminal region of rat *c-fos* peptide and is *c-fos* specific (Brennan *et al.*, 1992). The secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories). Fos immunoreactivity was visualized using diaminobenzidine (DAB). After section mounting, automated counting of stained nuclei was performed using an image analysis system (SeeScan Ltd, Cambridge, UK; Zhu *et al.*, 1995b). This analysis system captured 512×512 pixel images at a grey scale resolution with 256 levels. An object (nucleus) was automatically counted if its pixels were >25 grey scale levels darker than the darkest pixel surrounding it. This threshold difference was kept constant for all the imaged material. Processing and counting were completed with the experimenter blinded to which animals had been presented with the novel stimuli. Counts above threshold were obtained for rectangular areas (0.94×0.67 mm) from two non-adjacent coronal sections at each anterior-posterior level for each brain region (see Fig. 1 for the areas sampled). The boundaries of the various areas were determined from Paxinos & Watson (1986) and Shi & Cassell (1997).

Counts of Fos-stained nuclei in IC did not differ significantly with the novelty or familiarity of the stimuli (see Results). It was therefore possible to use counts from this earlier part of the auditory pathway to reduce between rat variability in the remaining data analysis; counts for each of the other areas were expressed as a ratio of the mean IC count for the same rat. To improve the normality of the distribution, these relative counts were subjected to a square root transformation before being entered into a repeat measures ANOVA with the factor for novelty/familiarity being measured between subjects, and that for area and the interaction of area and novelty/familiarity within subjects. Multiple frame counts within the same area and rat formed a substratum to this main analysis so that degrees of freedom remained

TABLE 2. Stained nuclei for the novel and familiar stimuli

Area	Stained nuclei (mean counts \pm SEM)	
	Novel	Familiar
IC	150.2 \pm 9.9	167.5 \pm 12.4
AudP	163.5 \pm 18.6	156.0 \pm 15.7
AudA	167.8 \pm 13.0	121.2 \pm 10.5
PRH	96.0 \pm 10.8	103.7 \pm 15.6
ENT	80.6 \pm 13.0	91.6 \pm 19.0
HPC	28.1 \pm 4.1	30.5 \pm 5.1
Amyg	58.5 \pm 7.3	60.9 \pm 8.2
TE2	116.6 \pm 14.8	125.4 \pm 17.1
PoRH	94.4 \pm 7.3	83.5 \pm 9.9

The counting frame size was 0.63 mm^2 . Note, in AudA, the counts for novel stimuli are substantially higher than those for familiar stimuli. The counts for PRH, AudP and HPC are the mean of all the sections collected in each of these regions for each condition.

appropriate to the numbers of rats and areas sampled (i.e. the main analysis used the mean value for each area for each rat). All statistical tests were two tailed and used a significance level of $P = 0.05$.

Results

The areas sampled were the IC, AudP (Te1), AudA (Te3), Amyg (lateral and basal lateral nuclei), area Te2, PRH, entorhinal cortex (ENT), PoRH and HPC (subfields CA1 and CA3, dentate gyrus, and subiculum). The PRH was sampled at three different levels (-4.3 , -5.3 and -6.3 mm behind bregma), and AudP at two levels (-4.3 and -5.3). The total number of sampled locations was 20 (Fig. 1). The medial geniculate nucleus was also sampled but, in common with findings for other sensory nuclei (Hunt *et al.*, 1987; Zhu *et al.*, 1996), few stained cells were found and the area was not analysed further. The mean counts for the sampled regions are given in Table 2.

Counts of Fos-stained nuclei in the earliest sampled stage of the auditory pathway, the IC, were subjected to ANOVA. The analysis showed that there was no significant effect of the novelty or familiarity of the stimuli on IC counts (mean counts for novel 150.2 ± 9.9 , for familiar 167.5 ± 12.4 ; ANOVA $F_{1,20} = 1.2$, $P = 0.29$). Therefore, IC counts were used to reduce between rat variability in the remaining data analysis by expressing counts for each of the other areas as a ratio of the mean IC count for the same rat; statistical analysis was conducted on the square root of these ratios. This analysis revealed a significant interaction between the factors area and novelty/familiarity (repeat measures ANOVA $F_{7,87} = 2.74$, $P = 0.011$), indicating that the effect of the relative familiarity of the stimuli varied between the areas. Further analysis (Fisher *t*-tests) demonstrated that novel stimuli produced significantly (designed comparison, $P < 0.01$) higher counts than familiar stimuli in AudA ($266 \pm 21/\text{mm}^2$ cf. $192 \pm 17/\text{mm}^2$), but not in PRH (designed comparison, $P > 0.1$), or in AudP, PoRH, Amyg, ENT or HPC (see Fig. 2). Analysis of counts at different anterior-posterior levels within PRH and for different subfields within the hippocampal formation did not show a significant difference at any of the individual locations. A separate analysis of the absolute counts (i.e. not expressed as a ratio of the IC counts) for AudA also revealed a significant difference between the counts for the novel and familiar stimuli (ANOVA $F_{1,20} = 7.78$, $P = 0.013$); there were no significant differences in the absolute counts in any other areas. Examples of Fos staining produced by novel and familiar sounds are shown in Fig. 3.

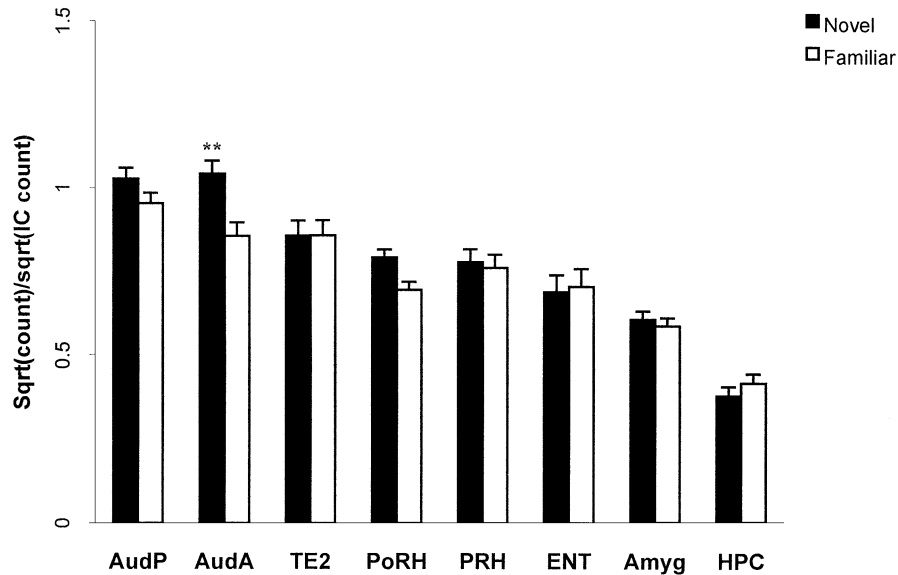


FIG. 2. Relative activation [square root of (mean Fos counts)/(mean IC Fos counts)] produced in different areas by novel and familiar sounds. Note the significantly greater activation in AudA produced by novel compared to familiar sounds. Abbreviations as for Fig. 1.

Discussion

The results indicate that novel complex sounds produce significantly greater neuronal activation than familiar sounds in rat AudA. In contrast to studies of visual recognition memory using the same strain of rat, PRH did not show significantly greater activation for novel than for familiar sounds. These results are consistent with the findings of recent lesion work in the dog and monkey: lesions of AudA produce impairment in delayed matching or nonmatching to sample tasks whereas lesions of PRH or hippocampus do not (Kowalska & Kuśmerek, 1997; Kowalska *et al.*, 1998, 2001; Saunders *et al.*, 1998). It should be noted that whereas differential activation by novel and familiar sounds necessitates a region being involved in the processing of auditory stimuli, a failure to find such differential activation allows no conclusion to be drawn concerning the involvement of a region in auditory processing.

The greater activation of the AudA produced by novel than by familiar sounds cannot readily be explained as resulting from a generalized increase in alertness or orienting to novelty in the rats that heard the novel sounds compared to those that heard the familiar sounds, as all counts were compared to those in the IC (an earlier part of the auditory pathway). Moreover, there was significantly increased activation in the auditory association cortex but not in AudP, PRH, Amyg or HPC, so that the increase was regionally specific and not generalized to all of the cortex, or even to all of the auditory cortex.

The lack of significant differential activation of PRH by the auditory stimuli used in the present experiment might imply that the role of the PRH in recognition memory differs for different modalities. However, alternative explanations must be excluded before this possibility is accepted.

The first possible explanation is that PRH receives less auditory than visual input, so that significant differential effects are less readily measured at the population level for auditory than for visual stimuli. However, it is not obvious from anatomical studies that there is less auditory input. PRH receives at least as many afferents designated as auditory as visual in the rat (Burwell & Amaral, 1998). The possibility that the auditory output goes to some unsampled region of PRH can be dismissed (unless the region within the PRH is

very circumscribed) as the sampled region spanned that receiving auditory input (Burwell & Amaral, 1998).

A second possible explanation is that the auditory system achieves a higher level of processing before the PRH is reached, so that the auditory association cortex is able to perform some function performed by the PRH in the visual system. Although this possibility cannot be excluded, visual association cortex (area Te2) is activated differentially by novel and familiar visual stimuli in correspondence with the activation of AudP (area Te3). Indeed, the magnitude of the differential activation is strikingly similar; the ratio of familiar to novel counts is 0.72 : 1 for the auditory stimuli used here and 0.75 : 1 for the visual stimuli used in previous experiments (Zhu *et al.*, 1996). This correspondence raises the possibility that processing for familiarity discrimination in the association cortices could be similar for the two modalities.

In such a case, a third possible explanation is suggested: the difference that might be crucial to the involvement of the PRH is in the nature of, or processing applied to, the individual auditory and visual stimuli. Recent evidence suggests that the PRH might have a specific role in the perception/categorization of visual stimuli as objects (Buckley & Gaffan, 1998; Murray & Bussey, 1999). If the PRH plays a similar role for auditory stimuli, a reason for its lack of differential activation in the present experiments could be that the sounds used were processed at the level of individual features and not processed at the more integrated level that would require the involvement of the PRH. A similar explanation could be used to explain the lack of perirhinal effects in the auditory lesion experiments where similar stimuli to those in the current study are used (Kowalska & Kuśmerek, 1997; Saunders *et al.*, 1998; Kowalska *et al.*, 2001). Such an explanation would not require the PRH to play a different role in auditory and visual recognition memory.

There was no significant difference in Fos staining produced by novel and familiar sounds in the HPC or PoRH. Moreover, the counts within the hippocampal formation were lower than those in other cortical areas. These results are consistent with findings using individual visual stimuli (Zhu *et al.*, 1995b; Wan *et al.*, 1999a). By contrast, components of the HPC and the PoRH are activated

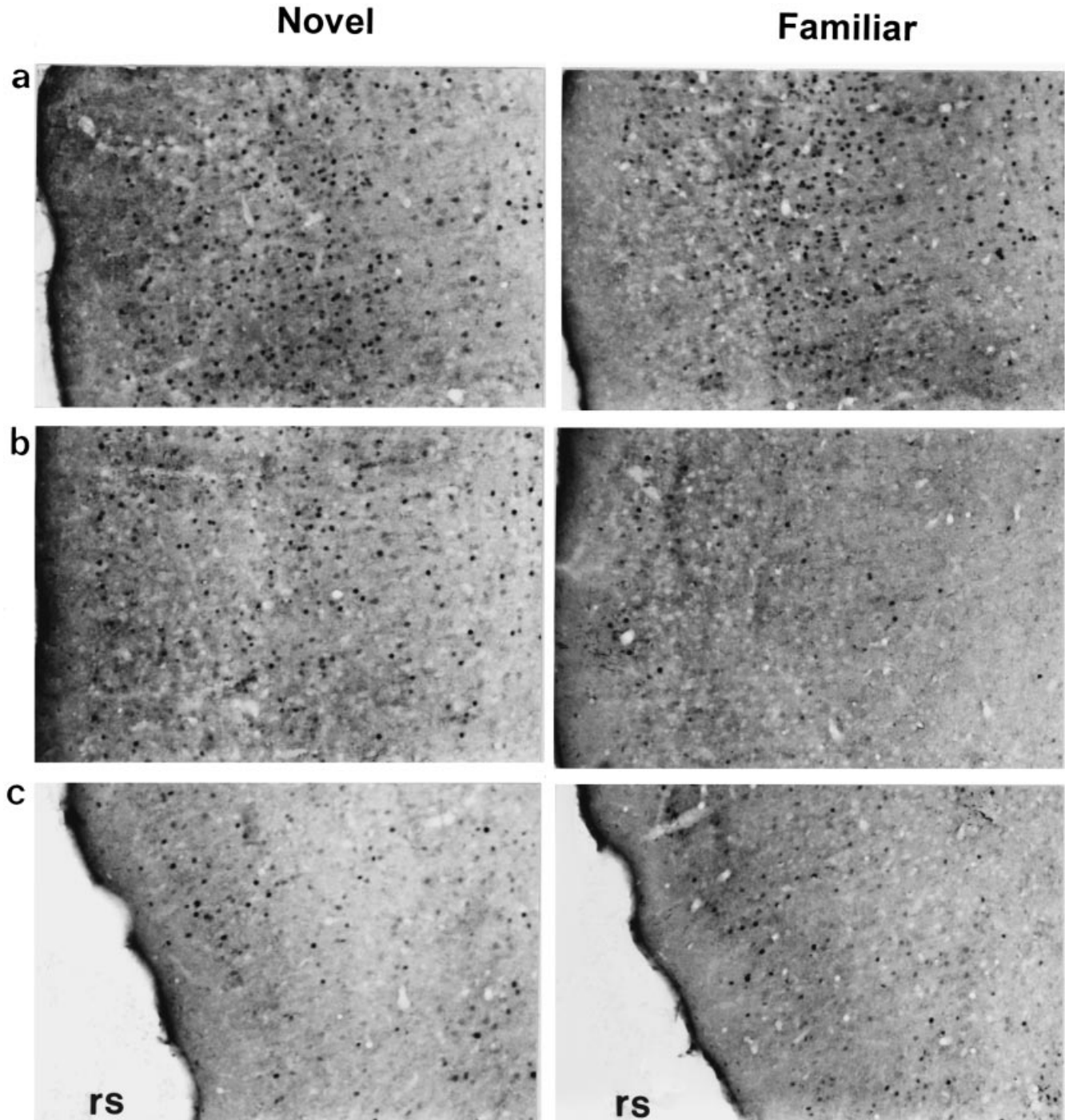


FIG. 3. Examples of Fos staining seen in different regions for the novel sounds (photomicrographs on the left) and for the familiar sounds (photomicrographs on the right). The regions are primary auditory cortex (AudP, area Te1; top), auditory association cortex (AudA, area Te3; middle) and PRH (PRH, area 36; bottom) all on sections 4.3 mm behind bregma. Note the greater density of stained nuclei for novel compared to familiar sounds in AudA. Magnification, 83 \times . rs, rhinal sulcus.

differentially by novel and familiar arrangements of familiar stimuli (Wan *et al.*, 1999a). It is not known whether novel and familiar patterns of familiar tones or other discrete sounds (e.g. 'hiss-clap-squeak' compared to 'squeak-hiss-clap') would result in differential activation in these regions. The lack of differential activation in the present experiment is consistent with findings using visual stimuli as the sets of sounds did not use such patterns.

Caution is required in comparing results of auditory recognition memory experiments across species. For example, there appear to be significant variations between the rat and the monkey in auditory connections into PRH. In particular, in monkeys, auditory connections into the PRH may be weaker than in the rat. Studies in monkeys (Suzuki, 1996a) indicate that the PRH receives a robust input from the unimodal visual association areas TE and TEO (Suzuki &

Amaral, 1994), whereas the PRH receives little direct input from the auditory association areas of the superior temporal gyrus. By contrast, in the rat (as mentioned above) the PRH receives greater auditory than visual input from adjacent sensory association cortices. However, in the monkey, the anterior PRH receives a more prominent projection from the most anterior aspects of the superior temporal gyrus (Suzuki & Amaral, 1994), and electrophysiological data indicate that cells in this part of the superior temporal gyrus respond to auditory as well as visual stimuli (Baylis *et al.*, 1987). A second possible route for auditory information going to the monkey PRH is provided by the strong projections that the PRH receives from area TH of the parahippocampal cortex; area TH receives inputs from auditory association areas of the superior temporal gyrus (Suzuki, 1996a). Therefore, in the monkey there is potentially more than one route that can be used by auditory information being processed in the temporal lobe. This raises the possibility that differing parahippocampal areas make significant contributions to auditory recognition memory in the monkey and, correspondingly, potentially complicates comparisons with findings from studies that use other species, including the rat.

In conclusion, novel sounds produced greater activation of the auditory association cortex than did familiar sounds, a result consistent with an important role for this cortex in the recognition memory process of familiarity discrimination. By contrast, the PRH was not activated differentially by novel and familiar sounds. The failure to find a difference in the activation of the PRH contrasts with results using visual stimuli. However, the different involvement of the PRH in the two modalities could relate to the processing of the stimuli employed in the studies. It has been suggested that the PRH is important for processing stimuli perceived as objects rather than as sets of individual features (Buckley & Gaffan, 1998; Murray & Bussey, 1999). This idea suggests that if the sounds used are processed solely at the level of component features, whereas the pictures used are processed both at the level of features and as an integrated percept (object), then the difference in perirhinal activation might be explained without invoking a fundamental difference between the two modalities in the processing underlying familiarity discrimination.

Acknowledgements

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Abbreviations

Amyg, amygdala (lateral and basal lateral nuclei); AudA, auditory association cortex (Te3); AudP, primary auditory cortex (Te1); ENT, entorhinal cortex; HPC, hippocampal formation; IC, inferior colliculus (external nucleus); PoRH, postrhinal cortex; PRH, perirhinal cortex (areas 35 and 36); Te2, visual association cortex.

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